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(54) Title: GENERATION OF CONDITIONAL YEAST MUTANTS, METHODS AND REAGENTS RELATED THERETO

(57) Abstract

The invention features a method for systematically generating mutant strains of obligate haploid and obligate diploid yeast, having condition–sensitive lethal mutations of a gene which is otherwise essential to cell viability under all conditions of cell growth. Conditional mutants make possible the analysis of physiological changes caused by inactivation of a gene or a gene product and can be used to address the function of any gene. In an exemplary embodiment, the method of the present invention can be generally characterized as follows: (i) providing a *Candida* strain whichis heterozygous for a loss–of–function mutation of an allele of an essential gene; and (ii) introducing a conditionally sensitive homolog of the gene, e.g., with conditional–sensitive protein or conditional–sensitive transcriptional regulatory sequence, at the functional allele by integrative transformation.

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*Generation of Conditional Yeast Mutants, Methods and Reagents Related Thereto*

**Background of the Invention**

The opportunistic human pathogen *Candida albicans* is a dimorphic asexual fungus. While this organism is usually found as a commensal of the human gut, it is also an agent of severe infection, especially of immuno-compromised individuals. The molecular mechanisms underlying pathogenicity, such as hyphal differentiation and production of virulence factors are of considerable interest. An understanding of these processes may reveal novel strategies for combating *Candida* infections.

Recently, recombinant technology for genetic analysis of this dimorphic yeast have been developed and applied to investigate diverse biological processes (Scherer and Magee, 1990 Microbiol Rev 54:226). Numerous *C. albicans* genes have been cloned and the nucleotide sequences determined. The use of such cloned sequences to analyze the function of proteins encoded by these genes by the introduction of defined mutations and deletions should allow detailed genetic and biochemical analysis of *C. albicans*. A major limitation to the widespread application of gene replacement techniques to study *C. albicans* has been the paucity of suitable auxotrophic host strains. As this organism is diploid, a two-step gene disruption procedure is generally needed in order to alter both alleles of a wild-type gene. Therefore, two different selectable markers are usually employed, imposing the need for a multiply-marked host strain. To circumvent this need for multiple auxotrophy, procedures which allow repeated use of the same selectable marker by alternate selection for and against the selectable phenotype have been employed. For example, the technique of Alani et al. (1987) Genetics 116:541, in which the selectable marker is lost by recombination between two flanking direct repeats, was successfully adapted for use in *C. albicans* (Gorman et al., 1991 Genetics 129:19; Fonzi and Irwin, 1993 Genetics 134:717). A second method employs the technique of co-transformation in which the selectable marker is located on a separate DNA fragment than the gene of interest (Rudolph et al., 1985 Gene 36:87). This method allows for independent loss and reuse of the selector.

*Candida spp*, in general, appear to be obligate diploids. The diploid genome and asexual life cycle of *C. albicans* have prevented those in the art from advancing a systematic approach for developing conditional mutants of genes which are essential to viability of *Candida* under all conditions. To date, Applicants are unaware of any published reports of the

successful generation of conditional alleles (e.g., temperature sensitive) of *Candida* genes which are otherwise essential to cell viability under all conditions.

It is therefore an object of the present invention to provide a means for generating conditional *Candida* mutants of essential genes.

## Summary of the Invention

The invention features a method for generating and identifying a strain carrying a lethal condition-sensitive mutation in a gene essential for survival.

In one aspect, there is provided a method for obtaining a condition-sensitive yeast mutant of an essential gene, the yeast being an obligate diploid. In general, the method involves beginning with a diploid yeast strain which is heterozygous for a loss-of-function mutation to the essential gene, wherein the strain is viable under both a permissive condition and a restrictive condition. This strain can be isolated from various sources, or generated by, e.g., homologous recombination. In one embodiment, the remaining wild-type allele of the essential gene is replaced with a condition-sensitive homolog thereof, e.g., which is lethal at the restrictive condition but not at the permissive condition, in order to generate a condition-sensitive yeast mutant. In other embodiments, at least a portion of the transcriptional regulatory sequence of the remaining wild-type allele of the essential gene is replaced with a conditional transcriptional regulatory sequence, e.g., conditionally inducible or conditionally repressible.

In another aspect, there is provided a method for obtaining a condition-sensitive yeast mutant of an essential gene, the yeast being an obligate haploid. As above, the single wild-type allele of the essential gene can be replaced with a condition-sensitive homolog thereof, e.g., which is lethal at the restrictive condition but not at the permissive condition, in order to generate a condition-sensitive yeast mutant. In other embodiments, the essential gene is replaced with a conditional transcriptional regulatory sequence. An exemplary obligate haploid is *Candida glabrata*.

In preferred embodiments, a condition-sensitive homolog of the essential gene replaces the wild-type allele by integrative transformation. For instance, integrative transformation can be accomplished with a circular vector including a recombination sequence represented by the general formula -A*-B-C*-D-, wherein A*,B, and C* represent nucleotide sequences that

hybridize under physiological conditions with the wild type allele (represented by the general formula -A-B-C-) and D represents a nucleotide sequence heterologous with respect to the wild type allele of the essential gene. Linearizing the vector by cutting within B will direct integration by homologous recombination.. Homologous recombination with the wild type allele produces a genomic structure represented by the general formula -A-B-C*-D-A*-B-C-. -A-B-C*- represents a condition-sensitive homolog of the wild type allele with the condition-sensitive mutation contained within C*. -A*-B-C- represents a non-expressed homolog of the wild type allele with A* containing a premature stop codon and in some cases a frameshift mutation.

D contains a marker for which there is negative selection. Applying negative selection induces homologous recombination between -AB-C*- and -A*-B-C- such that the repeat is resolved and D is eliminated from the genome. If homologous recombination occurs 5' to the mutation in A*, a nonfunctional allele of the general formula -A*-B-C-, will be generated and will not be recovered. If homologous recombination occurs between the mutation in A* and the mutation in C*, the wild type allele of the general formula -A-B-C- will be regenerated and recovered. If recombination occurs 3' to the mutation in C*, the condition sensitive allele of the general formula -A-B-C*- will be recovered. The last example represents a strain in which the wild type allele has been replaced with a mutant allele that confers a condition-sensitive phenotype.

In certain embodiments, the condition-sensitive homolog is a temperature sensitive homolog. In other embodiments, the condition-sensitive homolog is sensitive to one or more of the concentration of an ion, the concentration of a metal, pH or osmolarity, presence or absence of a carbon or nitrogen source.

An exemplary condition-sensitive homolog includes an inducible N-degron, such as a heat-inducible N-degron.

In still another embodiment, the expression of the remaining allele can be regulated by replacining all or a portion of the transcriptional regulatory sequences, e.g., promoter(s) and/or enhancer(s), with a conditional transcriptional regulatory sequence(s), such as an inducible promoter or enhancer or a repressible promoter or enhancer. In preferred embodiments, a condition-sensitive transcriptional regualtory sequence of the essential gene replaces the wild-type allele by integrative transformation.

In preferred embodiments, the diploid yeast is a *Candida* strain, such as may be selected from the group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida guilliermondii*, *Candida utilis* and *Candida rugosa*. In more preferred embodiments, the diploid yeast is *Candida albicans*.

The subject method can be used to identify essential genes which are required for cell wall biosynthesis.

In preferred embodiment, under the restrictive conditions, the condition-sensitive homolog causes cell lysis, mitotic catastrophe or growth arrest.

Another aspect of the present invention provides a condition-sensitive yeast mutant generated by the subject method.

Another aspect of the present invention relates to a method for identifying an essential gene of an obligate diploid yeast. According to this method, one starts with a diploid yeast strain which is heterozygous for a loss-of-function mutation to a gene, e.g., which strain is viable under both a permissive condition and a restrictive condition. The remaining wild-type allele of the gene is replaced with a condition-sensitive homolog thereof which results in a loss-of-function of the gene at the restrictive condition. Whether or not the loss-of-function is cytotoxic to the yeast under the restrictive condition is determined, wherein a cytotoxic phenotype for the loss-of-function is indicative that the gene is an essential gene.

The present invention further provides a method for identifying a compound having cytotoxic antifungal activity. The present invention contemplates the use of drug screening assays for detecting inhibitors of the function of an essential gene identified by the method set forth above in order to test compounds for their ability to inhibit the function of the essential gene. Such compounds can be used, e.g., as therapeutic agents to treat animals, as antiseptic agents to treat inanimate objects, as feed supplments for livestock, an in formulations for application to plants.

The present invention also provides pharmaceutical preparations formulated to include one or more compounds identified as having cytotoxic antifungal activity. Such compounds can be used as part of a method for treating or preventing an antifungal infection by being administered in an effective amount to an animal.

Still another aspect of the present invention provides a method for identifying a gene required for fungal virulence or pathogenecity in an animal comprising infecting an animal with a condition-sensitive yeast mutant generated as described herein, and measuring the virulence or pathogenecity of the condition-sensitive yeast mutant under restrictive conditions *in vivo*. As above, genes identified as being required for fungal virulence or pathogenecity in an animal can be used as part of a drug screening assay for identifying a compound for treating a fungal infection in an animal

Another aspect of the invention provides a method for obtaining a condition-sensitive yeast mutant of a gene essential to differentiation, the yeast being an obligate diploid. In general, the subject method utilizes a diploid yeast strain which is heterozygous for a loss-of-function mutation to the essential gene, which strain undergoes differentiation under both a permissive condition and a restrictive condition. The wild-type allele of the essential gene is replaced with a condition-sensitive homolog thereof which prevents differentiation under the restrictive condition, but not at the permissive condition, to generate a condition-sensitive yeast mutant. For example, the subject method can be used to identify genes required for hyphal morphogenesis. A similar method is contemplated for obtaining a condition-sensitive yeast mutant of a gene essential to differentiation, where the yeast is an obligate hapolid. As above, such genes are potential targets for drug screening assays generated to identify agents having antifungal activity.

In other embodiments, the subject condition-sensitive yeast mutants can be used identify a gene or gene product which is differentially expressed or whose stability is altered under restrictive conditions relative to permissive conditions. For example, the subject "indexing" method can be used to detect/identify mRNA transcripts (or cDNA) which have altered levels under restrictive conditions relative to permissive conditions.

For instance, transcripts isolated from the condition-sensitive yeast mutants under restrictive and permissive conditions can be subjected to "differential display" using such protocols as described by, e.g., Liang and Pardee (1992) Science 257:967-971, Chuang et al., (1993) J. Bacteriol. 175, 2026-2036, Lisitsyn et al. (1993) Science 259:946-951, and US Patents 5,837,468 and 5,807,680. For example, the method Chuang et al. identifies those genes which are expressed in an organism by identifying mRNA present using randomly-primed RT-PCR. By comparing pre-infection and post infection profiles, genes up and down

regulated during infection can be identified and the RT-PCR product sequenced and matched to library sequences.

Another indexing technique which can be used as part of the subject method is the *in vivo* expression technology (IVET). This technique is described by Camilli et al., (1994) PNAS 91:2634-2638. In this technique random chromosomal fragments of target organism are cloned upstream of a promoter-less reporter gene in a plasmid vector. The pool is introduced into a host and at various times after infection bacteria may be recovered and assessed for the presence of reporter gene expression. The chromosomal fragment carried upstream of an expressed reporter gene should carry a promoter or portion of a gene normally upregulated during infection, Sequencing upstream of the reporter gene allows identification of the up regulated gene.

Moreover, identification of genes or genes products which are differentially expressed/stable under restrictive versus permissive conditions yields additional information about its function and permits the selection of such sequence for further development as a screening target. Such embodiments of the subject method are useful for understanding mechanism of action of drugs which inhibit the function of an essential gene as descibed herein. Likwise, genes or gene products which are differentially expressed or whose stability is altered upon loss-of-function of the essential gene are themselves potential drug screening targets, e.g., as being in the pathway of the essential gene. Thus, another aspect of the present invention provides a method for identifying a compound having antifungal activity by providing a drug screening assay for detecting compounds which modulate the function of gene or gene product identified by the subject indexing method.

Other features and advantages of the present invention will be apparent from the following detailed description of the invention, the examples, and also from the appended claims.

**Brief Description of the Figures**

FIGURES 1A and 1B: Schematic representation of exemplary recombination vectors

FIGURE 2: Growth of *Candida albicans* cells on YPD at 23°C and 42°C.

A) Temperature sensitivity of URA3 prototrophic strains with a deletion of the gene of interest at one allele, and, at the second allele, a gene duplication with a stop codon in

one gene copy and an amino acid point mutation in the other. A URA3 gene lies between the two copies of the gene resulting in Uracil prototrophy. Uracil prototrophic strains: URA3/URA3 parent (CAF2); *URA3/URA3* parent (clinical isolate SC5314); *carhol Δ/carhol-3-URA3-carhol-STOP* (DIY-BL2-41 and 42); *carhol Δ/carhol-5-URA3-carhol-STOP* (DIY-BL2-45 and 48); *caram2 Δ/caram2-1-URA3-caram2-STOP* (DIY-BL2-53 and 55).

B) Temperature sensitivity of URA3 auxotrophic strains with a deletion of the gene of interest at one allele and an amino acid point mutation in the other at the second allele. Therese strains were derived from the strains shown in A) by selection of Uracil prototrophs on 5-FOA. Homologous recombination between the two copies of the gene of interest results in loss of the URA3 gene. Southern analysis revealed the regeneration of a single copy of the gene (not shown). Selection of temperature sensitive strains with a single gene copy was of interest to ensure stability of the desired mutations. Heterozygous null mutants; *carhol Δ/CARHOl* and *caram2 Δ/CARAM2* deleted for the genes of interest at only one allele grew as well as the parental wild-type strain. Uracil auxotrophic strains: *carhol Δ/carhol-3* (DIY-BL2-61), *carhol Δ/carhol-5* (DIY-BL2-70); *carhol Δ/CARHOl* (DIY-BL2-22); *caram2 Δ/caram2-1* (DIY-BL2-99); *caram2 Δ/CARAM2* (DIY-BL2-17); URA3/URA3 parent (CAF3-1).

Cultures were grown for 2 days shaking (220 RPM) at room temperature in 10ml of YPD containing 1M sorbitol and uridine in 50ml conical tubes. 0.5 x $10^4$ cells were added to 100 µl of YPD in microtiter dishes. Three ten-fold dilutions were made and 5 µl of each was spotted into YPD plates containing uridine to support growth or uracil prototrophic (URA/URA3) strains. Plates were incubated at either 23°C or 42°C for 2 days.

FIGURE 3: Regeneration of Uracil prototrophy in temperature sensitive mutants harboring a single copy of the temperature sensitive (ts) mutant gene of interest. Selected strains (from Fig. 2B) were chosen for reinsertion of the CaURA3 gene to obtain prototrophs. The plasmid pSCaURA3.4C7, which contains CaURA3 and one copy of the hisG repeat, was linearized

within the hisG sequence with Srf1 to target the plasmid to the deleted alleles of either CaRAM2 or CaRHO1 which contain a single hisG repeat.

Cultures were grown for 2 days shaking (220 RPM) at room temperature in a synthetic complete medium lacking uracil (SC-Ura). A 10 µl loop of cells was streaked onto SC-Ura agar plates. Plates were incubated at 23°C for 3 days or 42°C for 2 days. Prototrophic strains: Wild type (Sc5314 and CAF2); *carhol Δ/carhol-3* (DIY-BL2-61); *carhol Δ/carhol-5* (DIY-BL2-70); *caram2Δ/caram2-1* (DIY-BL2-99).

FIGURE 4: Growth of *C. albicans* yeast and mycelial forms at ambient and physiological temperatures.

A) Growth of colonies of *C. albicans* yeast cells in SC-Ura medium at 23°C and 37°C. Mutant strains *carho-3* (DIY-BL2-112), *carhol-5* (DIY-BL2-124); and *caram2-1* (DIY-BL2-99) form microcolonies of yeast cells (shown by arrows).

B) Growth of colonies of *C. albicans* mycelia on solid media containing 10% fetal calf serum (serum) and 14% agar at 23°C and 37°C. At ambient temperatures all strains grew as yeast-containing colonies of a similar diameter. At 37°C the wild type parent formed large exclusively mycelial colonies on serum plates. The three mutant strains formed very small colonies on serum (denoted by arrows) composed of both mycelia and yeast cells at 37°C.

For both A) and B) representative colonies in a field of colonies of similar size are shown.

FIGURE 5: RHO1 and RAM2 mutants have hyphal defects separable from growth defects.

FIGURE 6A: In a CaCAK1 heterozygote , the remaining WT allele of CaCAK1 is replaced with the CaURA3.PCK1.atgNATG.Ubi.RHCAK1 cassette.

FIGURE 6B: The depletion of CaCAK1 is evident in the above western when the promoter is repressed by glucose contianing media. The consequences associated with depletion of CaCAK1 on glucose plates at 42°C are illustrated.

FIGURE 7A: Shows the effect of depletion of CaRHO1.

FIGURE 7B: Shows CaRHO1 expression is controlled by the PCK1 promoter in glucose media.

FIGURE 7C: Shows loss of viability when CaRHO1 is depleted from cells by repressing the PCK1 promoter in glucose media.

**Detailed Description of the Invention**

*(i) Overview*

The invention features a method for systematically generating mutants of obligate diploids, such as *Candida* and other yeast strains, having condition-sensitive lethal mutations of a gene which is otherwise essential to cell viability under all conditions of cell growth. Conditional mutants make possible the analysis of physiological changes caused by inactivation of a gene or a gene product and can be used to address the function of any gene. The method of the present invention can be generally characterized as follows: (i) providing a obligate diploid strain which is heterozygous for a loss-of-function mutation of an allele of an essential gene; and (ii) introducing a conditionally sensitive homolog of the gene at the functional allele by integrative transformation.

In *Candida* and other obligate diploid yeast, the methods of the prior art have provided techniques for generating loss-of-function mutations of non-essential genes which are conditionally essential (e.g. essential under many but not all conditions). However, the method of the present invention can be used to generate these mutants, and as demonstrated in the appended examples, conditional loss-of-function mutants of truly essential genes.

The invention also features a method for systematically generating mutant strains of obligate haploid yeast having condition-sensitive lethal mutations of a gene which is otherwise essential to cell viability under all conditions of cell growth. In this aspect of the invention, there is introduced into the cell a conditionally sensitive homolog of the gene at the functional allele by integrative transformation.

The loss-of-function phenotype can include essential genes whose activity becomes conditioned on temperature, ion, metal, pH, osmolarity, and the like. The conditionally sensitive homolog can include mutations, or additional sequences (e.g. in the form of a fusion gene) which, under the non-permissive conditions, disrupts the normal bioactivity of the gene product. For example, the mutation(s) can conditionally disrupt protein-protein or protein-DNA interactions, catalytic activity (in the case of the gene encoding an enzyme), stability (folding, degradation), translocation, and the like.

Another aspect of the invention provides a strain of obligate diploid or haploid yeast, e.g., a *Candida* strain, having a condition-sensitive lethal mutation of an essential gene, e.g., which, under the appropriate conditions, is homozygous for loss-of-function of the essential gene.

Another aspect of the present invention is a method for determining if a gene is (i) an essential gene, and (ii) if it is an essential gene, whether its loss-of-function is cytostatic or cytotoxic to the cell. In the development of antifungal agents, it is desirable that the agent is cytotoxic, not merely cytostatic. Thus, essential genes which give rise to cytotoxic loss-of-function phenotypes are candidate targets for drug development assays.

Thus, another aspect of the present invention provides a drug screening assay for identifying agents which inhibit the activity of the wild-type form of an essential gene identified by the assay above. The invention also provides pharmaceutical preparations of such compounds, and methods for treating fungal infections using such preparations.

*(ii) Definitions*

For convenience, certain terms employed in the specification, examples, and claims are collected here.

An "essential gene", as used herein, is a gene whose gene product is required for cell viability under all circumstances such that loss-of-function of the gene under any circumstance

is lethal to the cell. In contrast, a "conditionally essential gene" is a gene whose function is required under most, but not all, conditions for cell viability. That is, there are conditions wherein a loss-of-function mutation of the conditionally essential gene are not lethal.

The term "loss-of-function" refers to mutations to a gene so as to render the gene product thereof to have substantially reduced activity, or preferably no activity relative to one or more functions of the corresponding wild-type gene. A "lethal loss-of-function" mutation is one wherein the activity of the mutant gene product is so impaired, relative to the wild-type gene, that the cell dies as a result of the mutation.

The term "homozygous for loss-of-function" of a gene refers to the loss-of-function of both alleles of a gene, or, more specifically, their gene products, either by the same or different mutations.

The effect of a "lethal mutation" cannot be reversed or overcome by shifting the organisms to permissive conditions. The selected genes and products thereof are therefore essential for survival of the organism under restrictive conditions. In contrast, strains having "non-lethal" or "static" mutations may grow very slowly or normally under restrictive conditions. The effect of a static mutation is reversible; organisms having static mutations resume metabolism and growth when shifted to permissive conditions.

A "conditional lethal mutation" results in a gene or a protein which is not functional under restrictive conditions.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is an genomic integrated vector, or "integrated vector", which can become integrated into the chromsomal DNA of the host cell. Another type of vector is an episomal vector, i.e., a nucleic acid capable of extra-chromosomal replication. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In the present specification, "plasmid" and "vector" are used interchangeably unless otherwise clear from the context.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer.

A "protein coding sequence" or a sequence which "encodes" a particular polypeptide, is a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

*(iii) Exemplary Embodiments*

The invention is based, in part, on the recognition that there are at least two types of strains (e.g., cells) which fail to grow under restrictive growth conditions: (a) strains with lethal mutations, and (b) strains with non-lethal or static mutations. In general, strains with lethal mutations are of greater interest for therapeutic research because a cytotoxic compound, for example, is generally more desirable than a cytostatic compound for use as an antifungal agent.

According to the invention, strains carrying lethal mutations must satisfy two criteria. First, when exposed to restrictive conditions for at least two growth cycles, the organism fails to grow. Examples of at least two growth cycles include at least 3, 4, 5, 7, 8, 10 or more growth cycles. Measurements of growth include RNA synthesis, DNA synthesis, protein synthesis, membrane morphology, success of division or reproduction, and levels of ATP. Examples of failure to grow therefore include serious membrane deformity, reduction or absence of DNA (or RNA or protein) synthesis, lysis, ATP depletion, unsuccessful division or reproduction, e.g., culminating in organism death.

The second criterion is that shifting such an organism from restrictive (non-permissive) conditions to permissive growth conditions for a period of time will not revive the organism or restore growth. That is, the *Candida* organism carries at least one lethal mutation, the product of which is irreversibly sensitive to the restrictive conditions. The gene carrying this mutation is essential for growth during the incubation period under the restrictive conditions.

According to one aspect of the invention, a strain carrying a non-lethal or static mutation is expressly avoided, i.e., not selected. A strain with a non-lethal or static mutation may fail to grow and reproduce under restrictive conditions, and yet will resume growth when shifted from restrictive to second permissive growth conditions. This resumption of substantially normal growth is generally apparent after two or more growth cycles under permissive conditions. In some cases, metabolism or growth may be initially slow during a transition period; in addition, growth may be slower than normal for several growth cycles. In either case, a strain with a static mutation does not satisfy the second criterion for lethal mutation as used herein.

The period of time will vary with the method of detecting growth or death, but is generally equivalent to a plurality of growth cycles (e.g., at least 2, 4, 6, 8, 10, 15, or 20 cycles). Depending on the strain and the difference between the restrictive and permissive conditions, growth may be delayed, or the rate of growth may increase during a transition period before stabilizing. Growth can be measured by methods known to those in the art, including expansion of colony cell mass, increased turbidity of a liquid cell culture suspension, cell or organism staining, DNA synthesis, and protein synthesis.

An illustrative embodiment of the present method for generating condition-sensitive *Candida* strains is illustrated in Figures 1A and 1B.

Merely to illustrate, the initial step requires that one allele of the target gene be deleted by gene replacement. To achieve this, the coding region or open reading frame (ORF) of interest can be replaced by a homologous recombination method, such as through the use of the "*Candida* URA3 blaster cassette" (described in appended examples) or the like. The resulting strain is heterozygous for loss-of-function of the targeted gene, though still viable. That is, a fully functional allele remains.

To replace the remaining copy of the targeted gene with a temperature sensitive version, an integration vector suitable for use in the *Candida* strain is generated. As described below, this can consist of the plasmid YIplac211 in which the *S. cerevisiae URA3* gene has been replaced with the *Candida* URA3 gene (this plasmid is referred to as pSCaURA3.2). This vector will now receive the *Candida* gene of interest which has 3 mutations relative to the wild-type gene. These mutations are the conditional mutation itself, a STOP codon (TAA) which can be marked by an Afl II restriction enzyme site, and a frameshift (FS) mutation immediately downstream of the STOP codon in the remote chance that there is read through of the STOP codon. The STOP and FS however, should be sufficient individually to effect the generation of a non-functional protein. To direct the integration event, it is important that there is a unique restriction site (present originally, or silently engineered into the sequence) located between the STOP/FS and the conditional mutation with which to linearize the integration plasmid. It is worth mentioning that the STOP/FS mutation can be located upstream or downstream of the conditional mutation.

Transformation of many species of *Candida* is achieved using such well-known protocols as the lithium acetate protocol. The transformation mixture is placed onto appropriate selective plates and transformants are recovered at the anticipated permissive temperature of the conditional mutation. As illustrated in Figure 1, the integration event will generate a duplication of the target gene. One copy will be non-functional as it possesses the STOP/FS and the other copy will harbor the conditional mutation and will be functional only at the permissive temperature. Consequently the conditional phenotype should be immediately apparent by screening transformants for growth at the permissive temperature but not at the non-permissive temperature. This fact facilitates the screening effort as such integration events seem to occur with a frequency of around 5%. At this stage, conditional mutants are screened by Southern blot analysis to ensure that the desired integration event has indeed been achieved. The final stage in the generation of the *C. albicans* temperature sensitive mutation is

to plate the mutant strains onto media containing 5-fluoro-orotic acid (5-FOA). This results in the eviction both of the CaURA3 and the inactive copy of the gene of interest. The desired colonies are *caURA3* minus and conditional for growth and should be analyzed by Southern to confirm the eviction of CaURA3 and one copy of the gene of interest.

As described in the examples set forth below, this strategy has been used to generate temperature sensitive strains of *C. albicans* that harbor mutations in the genes *CaRam2 (caram2-1)* and *CaRHO1 (carho1-3 & carho1-5)* that are equivalent to mutations recovered in the *S. cerevisiae* homologues. Figure 2 shows that these mutants recovered after the integrative transformation, but before 5-FOA treatment and therefore contain inactive and conditional copies of the gene of interest, are temperature sensitive at 41-43°C. To aid the recovery of these particular mutants, the transformation mixture was plated our onto glucose plates lacking uracil but supplemented with an osmotic stabilizer (1M sorbitol ) and transformants were recovered at room temperature.

Several types of conditional mutants and methods for producing them have been developed (Moir et al (1982) Genetics 100:565; Hill et al. (1987) Mol. Cell. Biol 7:2397; Yoshimatsu et al. (1989) Science 244:1346; Bartel et al. (1988) Cell 52:935; Zhang et al. (1991) PNAS 88:1511; Baim et al.(1991) PNAS 88:5072; Hu et al. (1987) Cell 48:555; and Riles et al. (1988) Genetics 118:601).

In addition to generating conditional *Candida* mutants based on analogy to known mutations in other yeast, the present invention also contemplates the generation of conditional mutations to essential genes by mutagenic techniques directly involving *Candida* genes. In an exemplary embodiment, the target (essential) gene is isolated and subjected to mutagenesis. For instance, the target gene can be subcloned from a *Candida* organism and mutagenized by any suitable method including, but not limited to, cassette mutagenesis, PCR mutagenesis (e.g., the fidelity of PCR replication can be reduced to induce mutation by varying $Mg^{2+}$ concentration, increasing the number of amplification cycles, altering temperatures for annealing and elongation, to yield random mutants), or physical or chemical mutagenesis of the entire gene or a portion thereof. Chemical mutagens include ethylmethanesulfonate (EMS), methylmethanesulfonate (MMS), methylnitrosoguanidine (NTG), 4-nitroquinoline-1-oxide (NQO), 2-aminopurine, 5-bromouracil, ICR 191 and other acridine derivatives, sodium bisulfite, ethidium bromide, nitrous acid, hydroxylamine, N-methyl-N'-nitroso-N-

nitroguanidine, and alkylating agents. Physical mutagens include ultraviolet radiation and x-rays.

The mutagenesis products can be used to construct a library of integration vectors including the mutant genes. The library of integrative vectors can be used to transfect *Candida* cells which have are heterozygous for loss-of-function of the target gene. Cell growth at under a desired permissive condition (such as a permissive temperature) allows for selection from the mutagenized gene library of those constructs retaining sufficient activity at that temperature. The viable cells are then shifted to growth under the desired non-permissive conditions. Cells which are unable to grow under those conditions are isolated. Those cells should now include a condition-sensitive allele of the target gene.

Another strategy for producing temperature-sensitive mutants, that does not require a search for a ts mutation in a gene of interest, is based on a portable, heat-inducible N-degron. The N-degron is an intracellular degradation signal whose essential determinant is a "destabilizing" N-terminal residue of a protein. A set of N-degrons containing different destabilizing residues is manifested as the N-end rule, which relates the in vivo half-life of a protein to the identity of its N-terminal residue. In eukaryotes, the N-degron consists of at least two determinants: a destabilizing N-terminal residue and a specific internal Lys residue (or residues) of a substrate. The Lys residue is the site of attachment of a multiubiquitin chain. Ubiquitin is a protein whose covalent conjugation to other proteins plays a role in a number of cellular processes, primarily through routes that involve protein degradation. For a description of exemplary heat-inducible N-degron modules which can be adapted for generating conditional mutants in *Candida*, see US Patents 5,705,387 and 5,538,862, and Dohmen et al. (1994) Science 263:1273-6.

The advantage of the heat-inducible N-degron (or "td") method is that it does not require an extensive, often unsuccessful search for a ts mutation in a gene of interest. If the protein of interest can tolerate an N-terminal extension, the corresponding td fusion is likely to be functionally unperturbed at permissive temperature. By contrast, low activity at permissive temperature is a common problem with conventionally derived ts proteins and is also expected to be a complication with proteins expressed from genes whose nonsense mutations are suppressed by a conditional suppressor tRNA.

A frequent problem with conditional phenotypes is the "phenotypic lag" that may occur between the imposition of nonpermissive conditions and the emergence of a relevant null

phenotype. The td method eliminates or reduces this problem, because the heat-induced activation of the conditional N-degron results in rapid disappearance of a td protein.

At present, the td method is confined to proteins of the cytosol and the nucleus--compartments where the N-end rule pathway is known to operate. However, the td concept should also be applicable to degrons in other compartments. Cytosolic degradation signals distinct from N-degrons and residing in either DHFR or other carriers should be feasible as well and may prove superior for certain applications. Varshavsky recently found (US Patent 5,763,212) that the heat induction of the Arg-DHFR$^{ts}$ N-degron is inhibited in the presence of methotrexate, a DHFR-specific, tightly binding substrate analog. The resulting possibility of controlling a degron with agents other than temperature can be used to construct new classes of conditional mutants in *Candida*.

In still another embodiment, the expression of the remaining allele can be regulated by replacining all or a portion of the transcriptional regulatory sequences, e.g., promoter(s) and/or enhancer(s), with a conditional transcriptional regulatory sequence(s), such as an inducible promoter or enhancer or a repressible promoter or enhancer. Exemplary yeast promoters, which regulate transcription of a gene by growth conditions, include are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Other exemplary conditional regulatory element include the glucose-repressible ADH2 promoter, and the repressible promoter of the yeast acid phosphatase gene (PHO5) which is derepressed by low concentrations of inorganic phosphate in the medium.

The subject method can be used to create condition-sensitive mutants of a variety of *Candida*, including *Candida albicans*, *Candida stellatoidea*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida guilliermondii*, or *Candida rugosa*.

Moreover, Applicants specifically contemplate that the subject method can be used to generate condition-sensitive mutants of other diploid fungus.

According to the invention, the transient disruption of gene function during the period of restrictive growth conditions results in cell death. Organism death can be macroscopically observed in a colony which has the same or reduced size over several growth cycles under the second permissive conditions. Light microscopy and staining can reveal cytological

deformations or other morphologies known by those in the art to be indicative of cell death. Under permissive or at least optimal conditions, protein synthesis occurs in a cell which is nominally alive. Dead cells are characterized, in part, by lysis or the absence of DNA, RNA, and protein synthesis.

Therapeutic agents can be developed from the identification of essential genes from *Candida*. Preferably, a gene product (e.g., a protein or an RNA molecule) identified as being essential by the methods disclosed herein is used in drug screening assays for development of antifungal agents. The disclosed gene selection methods establish that the gene product is essential for survival of the organism.

In this regard, the present invention provides a systematic and practical approach for the identification of candidate agents able to inhibit one or more of the essential gene's functions. In a general sense, the assays of the present invention evaluate the ability of a compound to inhibit the biological activity of the gene product of an essential gene, e.g., by inhibiting, as appropriate, such activities as protein-protein, protein-DNA, secretion, or an enzymatic activity associated with the product of the essential gene.

A variety of assay formats will suffice and, in light of the present disclosure, those not expressly described herein will nevertheless be comprehended by one of ordinary skill in the art. For instance, the assay can be generated in many different formats, and include assays based on cell-free systems, e.g. purified proteins or cell lysates, as well as cell-based assays which utilize intact cells. The assays may be formatted to evaluate such activity by adding the compound to a cell-free preparation (e.g., lysate or reconstituted protein mixture) including the product of the essential gene, or by contacting the agent with a cell which recombinantly expresses the essential gene.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays of the present invention which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins or with lysates, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an

alteration, as appropriate, of binding affinity with other proteins or changes in enzymatic properties of the molecular target.

Exemplary compounds which can be screened for activity against an essential gene identified by the subject method include peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries, such as isolated from animals, plants, fungus and/or microbes. In a preferred embodiment, the test agent is a small organic molecule having a molecular weight of less than about 2,000 daltons. For preferred assays of the instant invention, the assay is preferably repeated for a variegated library of at least 100 different test compounds, though preferably libraries of at least $10^3$, $10^5$, $10^7$, and $10^9$ compunds are tested. The test compound can be, for example, small organic molecules, and/or natural product extracts.

After identifying certain test compounds as potential antifungal agents, the practioner of the subject assay will continue to test the efficacy and specificity of the selected compounds both *in vitro* and *in vivo*. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, agents identified in the subject assay can be formulated in pharmaceutical preparations for *in vivo* administration to an animal, preferably a human.

The subject compounds selected in the subject method, or a pharmaceutically acceptable salt thereof, may accordingly be formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the compound, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations". Based on the above, such pharmaceutical formulations include, although not exclusively, solutions or freeze-dried powders of the compound in association with one or more pharmaceutically

acceptable vehicles or diluents, and contained in buffered media at a suitable pH and isosmotic with physiological fluids. In preferred embodiment, the compound can be disposed in a sterile preparation for topical and/or systemic administration. In the case of freeze-dried preparations, supporting excipients such as, but not exclusively, mannitol or glycine may be used and appropriate buffered solutions of the desired volume will be provided so as to obtain adequate isotonic buffered solutions of the desired pH. Similar solutions may also be used for the pharmaceutical compositions of compounds in isotonic solutions of the desired volume and include, but not exclusively, the use of buffered saline solutions with phosphate or citrate at suitable concentrations so as to obtain at all times isotonic pharmaceutical preparations of the desired pH, (for example, neutral pH).

**Exemplification**

The invention now being generally described will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

**Example 1**

**Methods for the generation of *caram2-1*, *carhol-3* and *carhol-5* temperature sensitive strains of *C. albicans***

**Methodology**

*Method 1*

*C. albicans* is a diploid and to generate temperature sensitive mutants requires that one allele be knocked out by gene replacement and the remaining allele to be replaced with a mutant version by integrative transformation. The mutations present in the integration vector are the conditional temperature sensitive mutation itself, a STOP codon (TAA) which can be marked by an Afl II restriction enzyme site, and a frameshift (FS) mutation immediately downstream of the STOP codon in the remote chance that there is read through of the STOP codon. The STOP and FS however should be sufficient individually to effect the generation of

a non-functional protein. The integration plasmid is directed to the desired locus by linearizing the plasmid with a unique restriction site located between the STOP/FS and the conditional mutation. The integration event will generate a non-functional allele containing the STOP-FS mutation and a temperature sensitive allele separated by the CaURA3 sequence. The temperature sensitive phenotype should therefore be immediately apparent. The vectors generated for this manipulation and the methods used to check transformed colonies are described.

*Method 2*

In a variation to Method 1, gene replacement of the remaining allele by integrative transformation is accomplished with an integration vector as described above, but which does not include the STOP-FS mutations. The integration plasmid is directed to the desired locus by linearizing the plasmid. The temperature sensitive phenotype will become evident upon treatment of the transformed cells 5-fluoroorotic acid (Boeke, J. D. et al. (1987) Methods Enzymol. 154: 164-175).

**Materials & Methods**

**Generation of knockout constructs for CaRHO1 and CaRAM2**

Using standard molecular biological procedures, the genomic loci for CaRAM2 and CaRHO1 (the sequence of which has been supplied previously) were used as templates for PCR to amplify the 5' and 3' non-coding regions thereby exactly deleting the ORF. The PCR fragments were cloned into pBluecript KS-. The CaURA3 blaster cassette on an BamHI/BglII fragment (received from W. Fonzi) was then inserted into BamHI & BglII sites of the recipient plasmids to generate the final knockout constructs for CaRAM2 and CaRHO1 (designated pSCaRAM2.21 and pSCaRHO1.6 respectively).

**Generation of *C. albicans* CAF3-1 strains heterozygous for either CaRAM2 or CaRHO1**

The CaRAM2 and CaRHO1 knockout constructs were excised by XhoI/NotI and Asp718 digests of their respective parent plasmids pSCaRAM2.21 and pSCaRHO1.6. The desired fragments were gel purified prior to being transformed into the *C. albicans* strain CAF3-1. The

method used for CAF3-1 transformation is a lithium acetate protocol (from U. of Minnesota *C. ablicans* web site: http://alces.med.umn.edu/*Candida*/liac.html). The transformation mixture is then plated onto selective (-Ura glucose) plates and incubated at 30°C for 3days. Individual transformants that appear are restreaked for singles and then preserved as a glycerol stock. To ensure that the correct integrative event has occurred, southern analysis was carried out on several colonies. Those colonies that exhibited the correct genotype were retained.

**Generation of the *caram2-1*, *carho1-3* and *carho1-5* temperature sensitive alleles and integration vectors.**

*1: CaURA3 integration vector.*

The vector used to integrate each of the temperature sensitive alleles is a modified derivative of YIplac211 (Gietz & Sugino, 1988, Gene, 74, 527-534) and is designated pSCaURA3.2. This vector was generated by replacing the *S. cerevisiae* URA3 gene with the *C. albicans* URA3 gene (on an AatII/Bst1107I fragment) which had been generated by PCR using the CaURA3 blaster cassette described above as a template. pSCaURA3.2 is the recipient vector for each of the temperature sensitive alleles of CaRAM2 and CaRHO1.

*2: The caram2-1 integration vector*

The *caram2-1* temperature sensitive allele possesses the mutation N143K. This mutation was initially reported in C. A. Omer et al, 1993 Biochemistry, 32, 5167-5176. The N143K mutation was introduced into the genomic CaRAM2 clone contained in the plasmid pSCaRAM2.13 by PCR using the Quikchange kit (Stratagene) according to the manufacturers instructions.

The sequence (with the mutation underlined) of the oligos used was

MutCR2#1: 5' CAGACCCCAAGAAACATCATGTTTGGTCGTATC 3'

MutCR1#2: 5' ACGACCAAACATGATGTTTCTTGGGGTCTGAAC 3'

The mutated allele was confirmed by sequencing and the resultant plasmid designated pSCaRAM2.16. To assist in marking the alleles of CaRAM2 a BamHI site was introduced in the 3' untranslated region of the gene. The oligos used for this PCR mutagenesis using the Quikchange kit (Stratagene) are as follows (the alteration is underlined):

CRAM2.13:5' CAATTGTTTAACTAGGATCCTCATTGTATACCACCGATATTAC 3'

CRAM2.14:5' TGGTATACAATGAGGATCCTAGTTAAACAATTGAAAAC 3'

The introduction of the BamHI site was confirmed by sequencing and this plasmid is designated pSCaRAM2.22. This mutated genomic CaRAM2 clone was subcloned into pSCaURA3.2 on an Asp718/HindIII fragment to generate the plasmid pSCaRAM2.29.

In order to be able to identify strains that are temperature sensitive immediately it is necessary to additionally introduce inactivating STOP and FRAMESHIFT (FS) mutations into the *caram2-1* sequence as described above. The Quikchange kit (Stratagene) was used according to the manufacturers instructions to introduce the STOP-FS mutations by PCR. The sequence (with the alteration underlined) of the oligos was

CRAMSTOPFS.1: 5' GAAGAGTTTAGTTCTTAAGTTTGTTGATTTGGAAAAAG 3'

CRAMSTOPFS.2: 5' CCAAATCAACAAACTTAAGAACTAAACTCTTCTAATTG 3'

An AflII site (CTTAAG) marked the actual STOP codon TAA and was used to screen for colonies that possessed this mutation. The *caram2-1* /STOP-FS and CaURA3 sequences were then confirmed by sequencing. This plasmid is the *caram2-1* integration plasmid and designated pSCaRAM2.30.

*3: The carho1-3 integration vector*

The *carho1-3* temperature sensitive allele possesses the mutation L59P (which is equivalent to L60P mutation found in *S. cerevisiae* RHO1 gene: Kamada *et al.* J. Biol. Chem. 1996, 271, 9193-9196 and additional unpublished information from D. E. Levin's laboratory). The L59P mutation was originally introduced into CaRHO1 cDNA in the plasmid PID018 generated by V. Damagnez by PCR using the Quikchange kit (Stratagene) according to the manufacturers instructions. The sequence of the oligos used, with the mutation underlined are:

CaRHO1.1 5' GGTAGAAAAGTTGAACCAGCATTATGGGATACTGC 3'

CaRHO1.2 5' CCCATAATGCTGGTTCAACTTTTCTACCATCAAC 3'

The mutated allele was confirmed by sequencing and the resultant plasmid designated pSCaRHO1.1. The mutated CaRHO1 ORF was then subcloned into pSCaRHO1.3 cut PacI/HincII to generate pSCaRHO1.8 which represents a genomic *carho1-3* clone harboring the L59P mutation. The mutated genomic *carho1-3* clone was then subcloned into pSCaURA3.2 on an EcoRV/HindIII fragment to generate the plasmid pSCaRHO1.13. In order to be able to identify strains that are temperature sensitive immediately it is necessary to additionally introduce inactivating STOP and FRAMESHIFT (FS) mutations into the *carho1-3* sequence as described above. The Quikchange kit (Stratagene) was used according to the manufacturers instructions to introduce the STOP-FS mutations by PCR. The sequence (with the alteration underlined) of the oligos was

CaRHOSTOPFS.1: 5' CTGAACTTCGTACTTAAGTTAGTCATTGTCGGTG 3'

CaRHOSTOPFS.2: 5' CACCGACAATGACTAACTTAAGTACGAAGTTCAG 3'

An AflII (CTTAAG) site marked the actual STOP codon TAA and was used to screen for colonies that possessed this mutation. The *carho1-3* /STOP-FS and CaURA3 sequences were then confirmed by sequencing. This plasmid is the *carho1-3* integration plasmid and designated pSCaRHO1.15.

*4: The carho1-5 integration vector*

The *carho1-5* temperature sensitive allele possesses the mutation G120C (which is equivalent to G121C mutation found in *S. cerevisiae* RHO1 gene: Kamada *et al.* J. Biol. Chem. 1996, 271, 9193-9196 and additional unpublished information from D. E. Levin's laboratory). The G120C mutation was originally introduced into CaRHO1 cDNA in the plasmid PID018 generated by V. Damagnez by PCR using the Quikchange kit (Stratagene) according to the manufacturers instructions. The sequence of the oligos used, with the mutation underlined, are:

CaRHO1.3: 5' CCAATCATTTTAGTTTGTTGTAAATCTGATTTAAG 3'

CaRHO1.4: 5' TCAGATTTACAACAAACTAAAATGATTGGAACAC 3'

The mutated allele was confirmed by sequencing and the resultant plasmid designated pSCaRHO1.2. The mutated CaRHO1 ORF was then subcloned into pSCaRHO1.3 cut PacI/HincII to generate pSCaRHO1.7 which represents a genomic *carho1-5* clone harboring

the G120C mutation. The mutated genomic *carho1-5* clone was then subcloned into pSCaURA3.2 on an EcoRV/HindIII fragment to generate the plasmid pSCaRHO1.12. Then as described above for pSCaRHO1.15 STOP-FS mutations were introduced using the Quikchange kit (Stratagene) into the carho1-5 allele using he primers CaRHOSTOPFS.1 & CaRHOSTOPFS.2 detailed above. An AflII (CTTAAG) site marked the actual STOP codon TAA and was used to screen for colonies that possessed this mutation. The *carho1-5* /STOP-FS and CaURA3 sequences were then confirmed by sequencing. This plasmid is the *carho1-5* integration plasmid and designated pSCaRHO1.14.

**Transformation of the CaRAM2 and CaRHO1 integration plasmids**

Transformation of integration plasmids into the *C. albicans* strain CAF3-1.

For transformation each of the plasmids were linearized with the following enzymes to target their integration to the desired locus:

pSCaRAM2.30 (*caram2-1*) 10µg was linearized with BstXI

pSCaRHO1.14 (*carho1-5*) 10µg was linearized with BsmFI

pSCaRHO1.15 (*carho1-3*) 10µg was linearized with BsmFI

Transformation of *C. albicans* is achieved using a lithium acetate protocol (available from the Univ. of Minnesota *C. ablicans* web site: http://alces.med.umn.edu/*Candida*/liac.html). The transformation mixture is plated onto appropriate selective plates (-Ura glucose +1M sorbitol) plates that were also supplemented with 1M sorbitol which may stabilize the cells if the introduced mutations rendered them osmotically sensitive. The plates were incubated at room temperature (RT) for 5-7 days prior to picking colonies. Each of the integration plasmids gave rise to numerous colonies.

**Selection of temperature sensitive mutants**

Isolated colonies were streaked for singles prior to patching onto a -Ura Glc 1M sorbitol plates . The patched plates served as templates for replica plates of -Ura Glc. The replica plates were then placed in a 42°C incubator or left at RT. Patches of cells that either failed to grow at 42°C or appeared yellow were saved and glycerol stocks were made. In each case between 70-150

colonies were screened for inability to grow at 42°C. Temperature sensitive colonies were recovered at a rate of 2-3%.

**Southern validation of the integration events into the CaRAM2 and CaRHO1 loci.**

Temperature sensitive strains were grown on selective plates supplemented with 1M sorbitol and were harvested after 5days growth at room temperature. Genomic DNA was harvested using standard protocols. 2µg of the genomic DNA was cut using the following enzymes

For *caram2-1* strains: PstI/Bst1107I

For *carho1-3* & carho1-5 strains: HindIII/EcoRV

The digested DNA was resolved on a 1% agarose gel, and was then southern blotted onto Nytran membranes. The membranes were hybridized overnight with $^{32}P$ labeled DNA probes of either the 5' untranslated region of CaRAM2 or the 5' untranslated region of CaRHO1 in Church buffer at 42°C. The membranes were then washed with 0.2xSSC, 0.1%SDS and exposed to film. Those CAF3-1 derivatives which had the appropriate pattern were retained.

**Eviction of the STOP-FS mutated alleles of CaRAM2 and CaRHO1.**

As explained above the integration of the temperature sensitive STOP-FS alleles of CaRAM2 and CaRHO1 at the genomic locus of interest generates a duplication of the gene which flank the CaURA3 marker . This arrangement allows the eviction of the CaURA3 gene along with one allele when cells are grown on 5-FOA plates. *caram2-1*, *carho1-3* and *carho1-5* strains which appeared correct by southern blot analysis were grown overnight in rich media (YPD supplemented with uridine) prior to being plated out on 1M Sorbitol plates also containing 2% glucose, 5-FOA at 1mg/ml and uridine at 25µg/ml. Plates were incubated at room temperature for 5 days. Colonies showing temperature sensitivity at 42°C were preserved as glycerol stocks.

| Strains | genotype |
|---|---|
| SC5314 | *URA3/URA3* |
| CAF2 | *URA3/URA3Δ* |
| CAF3-1 | *URA3Δ/URA3Δ* |
| DIY-BL2-007 | *CARAM2/caram2Δ::hisgURA3hisg URA3Δ/URA3Δ* |
| DIY-BL2-008 | *CARHO1/carho1Δ::hisgURA3hisg URA3Δ/URA3Δ* |
| DIY-BL2-017 | *CARAM2/caram2Δ::hisg URA3Δ/URA3Δ* |
| DIY-BL2-022 | *CARHO1/carho1Δ::hisg URA3Δ/URA3Δ* |
| DIY-BL2-041 | *carho1Δ::hisg/carho1-3-URA3-carho1-STOP URA3Δ/URA3Δ* |
| DIY-BL2-042 | *carho1Δ::hisg/carho1-3-URA3-carho1-STOP URA3Δ/URA3Δ* |
| DIY-BL2-045 | *carho1Δ::hisg/carho1-5-URA3-carho1-STOP URA3Δ/URA3Δ* |
| DIY-BL2-048 | *carho1Δ::hisg/carho1-5-URA3-carho1-STOP URA3Δ/URA3Δ* |
| DIY-BL2-053 | *caram2Δ::hisg/caram2-1-URA3-caram2-1-STOP URA3Δ/URA3Δ* |
| DIY-BL2-055 | *caram2Δ::hisg/caram2-1-URA3-caram2-1-STOP URA3Δ/URA3Δ* |
| DIY-BL2-061 | *carho1Δ::hisg/carho1-3 URA3Δ/URA3Δ* |
| DIY-BL2-070 | *carho1Δ::hisg/carho1-5 URA3Δ/URA3Δ* |
| DIY-BL2-099 | *caram2Δ::hisg/caram2-1 URA3Δ/URA3Δ* |
| DIY-BL2-100 | *caram2Δ::hisg/caram2-1 URA3Δ/URA3Δ* |
| DIY-BL2-112 | *carho1Δ::hisg:: URA3/carho1-3 URA3Δ/URA3Δ* |
| DIY-BL2-124 | *carho1Δ::hisg:: URA3/carho1-5 URA3Δ/URA3Δ* |
| DIY-BL2-148 | *caram2Δ::hisg::URA3/caram2-1 URA3Δ/URA3Δ* |

**Example 2**

***Methods for the generation of conditional CaPCK1 promoter to assess the essentiality of a gene in the obligate diploid C. albicans***

The C. albicans PCK1 promoter was cloned by Leuker et al. 1997 Gene 192:235-240. This promoter is repressed by glucose and derepressed by gluceoneogenic carbon sources such as succinate and casamino acids. Heterologous genes placed under the control of the PCK1 promoter are similarily regulated by the nature of the carbon source in the medium. C. albicans is a diploid organism for which there is no known sexual phase which makes it difficult to determine whether a gene is essential. The use of a conditional promoter to switch off expression of a gene in a particular medium allows studies to determine whether C. albicans cells can survive in the absence of a particular gene.

Several groups, including Yeast Genetics at Mitotix, have utilised the PCK1 promoter for this purpose but it is apparent that the PCK1 promoter is not completely repressed in glucose containing media (see Stoldt et al. (1997) EMBO J. 16:1982-1991 and Figure 6B). Here we describe a number of strategies which enhance the usefulness of the PCK1 promoter.

In one embodiment, we seek to reduce the translation efficiency of the heterologous gene expressed from the PCK1 promoter. This can be achieved by

1. the introduction of out-of-frame ATGs upstream of the desired ORF. This reduces the production of the desired ORF (see, for example, Hinnebusch & Liebman, 1991, CSH. The Molecular & Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis & Energetics, Chapter 11); or
2. the use of a leader sequence upstream of the starting ATG which forms a stem:loop structure. Such a structure hinders the initiation of translation thereby reducing production of the desired ORF (see, Hinnesbusch & Liebman, supra)

In another embodiment, we seek to destabilize the mRNA encoding the heterologous gene expressed from the PCK1 promoter. This can be done by incorporating 2 distinct sequence motifs in the 3' untranslated region of the gene of interest (see Zubiaga et al., 1995, Mol. Cell. Biol., 15, 2219-30; and Marshall et al, 1996, BBA, 1308, 88-92). These motifs are the AUUUA element (present in the nonamer UUAUUUAUU) and the GAUG element.

In still another embodiment, we seek to destabilize the protein encoded expressed from the PCK1. This can be done by employing the N-end rule (Bachmair & Varshavsky, Cell, 1989, 56, 1019-1032) which targets the protein of interest for degradation by the ubiquitin pathway. The effect of including the destabilising residue R at the N-terminus of CaCAK1 is shown in Fig 6. Note that for this example the lacI sequence, which normally supplies the K residue which receives the ubiquitin moiety was not included. An internal lysine residue in the CaCAK1 sequence fulfills this requirement.

In yet another embodiment, we seek to improve the PCK1 promoter. The PCK1 promoter is not repressed fully in glucose containing media. It may be possible to repress the promoter more completely by incorporating an additional repression element that functions independently of glucose concentration. Examples of such elements include the haploid specific gene (HSG) element recently also found to function in C. albicans (Hull & Johnson, Science, 1999, 285, 1271). When 3 HSG elements were incorporated into the ADH promoter they inactivated this promoter (Hull & Johnson, supra). An HSG element used individually may provide intermediate levels of repression more suitable for regulating the PCK1 promoter.

It may be necessary to combine one of more of these possibilities to obtain a totally regulatable PCK1 promoter with any particular gene. Evidence is presented in the following figures of the usefulness of the approaches described above to regulate genes.

Figure 6A show that in a CaCAK1 heterozygote, the remaining WT allele of CaCAK1 is replaced with the CaURA3.PCK1.atgNATG.Ubi.RHCAK1 cassette. The CaCAK1 protein has been made a substrate of the N-end rule by fusing the ubiquitin protein and the amino acids RH N-terminally to the CaCAK1 ORF. This cassette is placed under the control of the PCK1 promoter. There is also an upstream, out of frame ATG, which reduces production of the desired ORF. The ATG.UbiGG.RHCaCAK1 when expressed is immediately processed by a ubiquitin hydrolase which recognises the UbiGG sequence and removes this element exposing CaCAK1 with an N-terminal destabilising amino acid arginine (R). RHCaCAK1 is then degraded by the ubiquitin system.

Figure 6B illustrates that the depletion of CaCAK1 is evident in the above western when the promoter is repressed by glucose contianing media. The consequences associated with depletion of CaCAK1 on glucose plates at 42°C are illustrated.

Figure 7A shows the effect of depletion of CaRHO1. In this PCK1.CaRHO1 construct, a short leader sequence has been inserted between the PCK1 promoter and the ATG of

CaRHO1. This leader sequence is shown below and has the potential to form a hairpin loop structure as indicated. The PCK1 promoter is underlined. The ATG of CaRHO1 is also indicated.

Figure 7B shows that CaRHO1 expression can be controlled by the PCK1 promoter in glucose media. Lane 1, levels of CaRHO1 and PGK1 in WT CAF2 cells grown in YPD, Lane 2 levels of CaRHO1 and PGK1 in cells dependent on CaRHO1 expression from the PCK1 promoter in YPCAA (PCK1 promoter on). Lane3, Level of CaRHO1 in cells dependent on CaRHO1 expression from the PCK1 promoter after 4hrs in Glc media (PCK1 promoter Off), Lane4, after 8hrs in Glc media (PCK1 promoter Off), Lane5, after 11hrs in Glc media (PCK1 promoter Off), Lane6, after 14hrs in Glc media (PCK1 promoter Off).

Figure 7C shows loss of viability when CaRHO1 is depleted from cells by repressing the PCK1 promoter in glucose media.

All of the above-cited references and publications are hereby incorporated by reference.

**Equivalents**

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention.

We Claim:

1. A method for obtaining a condition-sensitive yeast mutant of an essential gene, the yeast being an obligate diploid, comprising:

   (i) providing a diploid yeast strain which is heterozygous for a loss-of-function mutation to the essential gene, which strain is viable under both a permissive condition and a restrictive condition; and

   (ii) replacing the wild-type allele of the essential gene with a condition-sensitive homolog thereof which is lethal at the restrictive condition, but not at the permissive condition, to generate a condition-sensitive yeast mutant.

2. The method of claim 1, wherein the condition-sensitive homolog of the essential gene replaces the wild-type allele by integrative transformation.

3. The method of claim 1, wherein the condition-sensitive homolog is a temperature sensitive homolog.

4. The method of claim 1, wherein the condition-sensitive homolog is sensitive to one or more of the concentration of an ion, the concentration of a metal, pH or osmolarity.

5. The method of claim 1, wherein the condition-sensitive homolog includes an inducible N-degron.

6. The method of claim 5, wherein the inducible N-degron is a heat-inducible N-degron.

7. The method of claim 1, wherein the diploid yeast is a *Candida* strain.

8. The method of claim 7, wherein the diploid yeast is selected from the group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida guillermondii*, and *Candida rugosa*.

9. The method of claim 7, wherein the diploid yeast is *Candida albicans*.

10. The method of claim 2, wherein the condition-sensitive homolog replaces the wild-type allele by integrative transformation with a vector including a recombination sequence represented by the general formula -A*-B-C*-D-, wherein A*,B, and C* represent nucleotide sequences that hybridize under physiological conditions with the wild type

allele (represented by the general formula -A-B-C-) and D represents a nucleotide sequence which is heterologous with respect to the wild type allele,

such that homologous recombination with the wild type allele produces a genomic structure represented by the general formula -A-B-C*-D-A*-B-C-, wherein -A-B-C*- represents a condition-sensitive homolog of the wild type allele with the condition-sensitive mutation contained within C*, and -A*-B-C- represents a non-expressed homolog of the wild type allele with A*.

11. The method of claim 10, wherein -A*-B-C- introduces a premature stop codon into the sequence -A-B-C-.

12. The method of claim 1, wherein the essential gene is required for cell wall biosynthesis.

13. The method of claim 1, wherein the condition-sensitive yeast mutant ceases cell division at the restrictive condition.

14. The method of claim 13, wherein the condition-sensitive yeast mutant ceases cell division after no more than 5 growth cycles.

15. The method of claim 1, wherein, under the restrictive conditions, the condition-sensitive homolog causes membrane deformity, reduction or absence of DNA, RNA or protein synthesis or ATP depletion.

16. The method of claim 1, wherein, under the restrictive conditions, the condition-sensitive homolog causes cell lysis.

17. The method of claim 1, wherein, under the restrictive conditions, the condition-sensitive homolog causes mitotic catastrophe.

18. The method of claim 1, wherein growth of the condition-sensitive yeast mutant is measured by changes in colony cell mass, changes in turbidity of a liquid cell culture, changes in cell staining, changes in DNA synthesis, or changes in protein synthesis.

19. A condition-sensitive yeast mutant generated by the method of claim 1.

20. A method for obtaining a condition-sensitive yeast mutant of an essential gene, the yeast being an obligate diploid, comprising:

(i) providing a diploid yeast strain which is heterozygous for a loss-of-function mutation to the essential gene, which strain is viable under both a permissive condition and a restrictive condition; and

(ii) replacing a transcriptional regulatory sequence of the wild-type allele of the essential gene with a condition-sensitive transcriptional regulatory sequence which induces or represses expression of the gene under the restrictive condition, but not at the permissive condition, to generate a condition-sensitive yeast mutant with respect to expression of the essential gene.

21. The method of claim 20, wherein the condition-sensitive transcriptional regulatory sequence replaces the wild-type allele by integrative transformation.

22. The method of claim 20, wherein the condition-sensitive transcriptional regulatory sequence is sensitive to one or more of the concentration of an ion, the concentration of a metal, the concentration of a metabolite, pH or osmolarity.

23. The method of claim 20, wherein the diploid yeast is a *Candida* strain.

24. The method of claim 23, wherein the diploid yeast is selected from the group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida guillermondii*, and *Candida rugosa*.

25. The method of claim 20, wherein the diploid yeast is *Candida albicans*.

26. The method of claim 20, wherein the essential gene is required for cell wall biosynthesis.

27. The method of claim 20, wherein the condition-sensitive yeast mutant ceases cell division at the restrictive condition.

28. The method of claim 27, wherein the condition-sensitive yeast mutant ceases cell division after no more than 5 growth cycles.

29. The method of claim 20, wherein, under the restrictive conditions, the change in expression level of essential gene by the condition-sensitive transcriptional regulatory sequence causes membrane deformity, reduction or absence of DNA, RNA or protein synthesis or ATP depletion.

30. The method of claim 20, wherein, under the restrictive conditions the change in expression level of essential gene by the condition-sensitive transcriptional regulatory sequence causes cell lysis.

31. The method of claim 20, wherein, under the restrictive conditions, the change in expression level of essential gene by the condition-sensitive transcriptional regulatory sequence causes mitotic catastrophe.

32. The method of claim 20, wherein growth of the condition-sensitive yeast mutant is measured by changes in colony cell mass, changes in turbidity of a liquid cell culture, changes in cell staining, changes in DNA synthesis, or changes in protein synthesis.

33. A condition-sensitive yeast mutant generated by the method of claim 20.

34. A method for obtaining a condition-sensitive yeast mutant of an essential gene, the yeast being an obligate haploid, comprising

(i) providing a haploid yeast strain which is viable under both a permissive condition and a restrictive condition; and

(ii) replacing the wild-type allele of the essential gene with a condition-sensitive mutant thereof, or a having condition-sensitive transcriptional regulatory seqence, which is lethal at the restrictive condition, but not at the permissive condition, to generate a condition-sensitive yeast mutant.

35. A condition-sensitive yeast mutant generated by the method of claim 34.

36. A method for identifying an essential gene of an obligate diploid yeast, comprising:

(ii) providing a diploid yeast strain which is heterozygous for a loss-of-function mutation to a gene, which strain is viable under both a permissive condition and a restrictive condition;

(ii) replacing the wild-type allele of the gene with a condition-sensitive homolog thereof which results in a loss-of-function or loss-of-expression of the gene at the restrictive condition; and

(iii) determining if, under the restrictive condition, the loss-of-function is cytotoxic to the yeast,

wherein, if the loss-of-function is cytotoxic to the yeast then the gene is an essential gene.

37. A method for identifying a compound having cytotoxic antifungal activity comprising,

(i) providing a drug screening assay for detecting inhibitors of the function of an essential gene identified by the method according to claim 36; and

(ii) testing, in the drug screening assay, the ability of a test compound to inhibit the function of the essential gene.

38. A method for treating or preventing an antifungal infection comprising administering to an animal an effective amount of a compound identified according to the method of claim 37 as having cytotoxic antifungal activity.

39. The method of claim 37, comprising the further step of formulating a pharmaceutical preparation including one or more compounds identified as having cytotoxic antifungal activity.

40. A pharmaceutical preparation formulated according to the method of claim 39.

41. A method for identifying an essential gene of an obligate haploid yeast, comprising:

(ii) providing a haploid yeast strain which is viable under both a permissive condition and a restrictive condition;

(ii) replacing the wild-type allele of a gene with a condition-sensitive homolog thereof which results in a loss-of-function or loss-of-expression of the gene at the restrictive condition; and

(iii) determining if, under the restrictive condition, the loss-of-function is cytotoxic to the yeast,

wherein, if the loss-of-function is cytotoxic to the yeast then the gene is an essential gene.

42. A method for identifying a compound having cytotoxic antifungal activity comprising,

(i) providing a drug screening assay for detecting inhibitors of the function of an essential gene identified by the method according to claim 41; and

(ii) testing, in the drug screening assay, the ability of a test compound to inhibit the function of the essential gene.

43. A method for treating or preventing an antifungal infection comprising administering to an animal an effective amount of a compound identified according to the method of claim 42 as having cytotoxic antifungal activity.

44. The method of claim 42, comprising the further step of formulating a pharmaceutical preparation including one or more compounds identified as having cytotoxic antifungal activity.

45. A pharmaceutical preparation formulated according to the method of claim 44.

46. A method for identifying a gene required for fungal virulence or pathogenecity in an animal comprising infecting an animal with a condition-sensitive yeast mutant of claim 33 or 35, and measuring the virulence or pathogenecity of the condition-sensitive yeast mutant under restrictive conditions *in vivo*.

47. A method for identifying a compound for treating a fungal infection in an animal comprising,

(i) providing a drug screening assay for detecting inhibitors of the function of a gene identified as being required for fungal virulence or pathogenecity by the method according to claim 46; and

(ii) testing, in the drug screening assay, the ability of a test compound to inhibit the function of the essential gene.

34. A method for treating or preventing an antifungal infection comprising administering to an animal an effective amount of a compound identified as an inhibitor in the method of claim 33.

35. The method of claim 33, comprising the further step of formulating a pharmaceutical preparation including one or more compounds identified as inhibitors of the essential gene.

36. A pharmaceutical preparation formulated according to the method of claim 35.

37. A method for obtaining a condition-sensitive yeast mutant of a gene essential to differentiation, the yeast being an obligate diploid, comprising:

(i) providing a diploid yeast strain which is heterozygous for a loss-of-function mutation to the essential gene, which strain undergoes differentiation under both a permissive condition and a restrictive condition; and

(ii) replacing the wild-type allele of the essential gene with a condition-sensitive homolog thereof which prevents differentiation under the restrictive condition, but not at the permissive condition, to generate a condition-sensitive yeast mutant.

38. The method of claim 37, wherein the condition-sensitive yeast mutant cannot undergo hyphal morphogenesis under the restrictive condition

39. A method for obtaining a condition-sensitive yeast mutant of a gene essential to differentiation, the yeast being an obligate haploid, comprising:

(i) providing a haploid yeast strain which undergoes differentiation under both a permissive condition and a restrictive condition; and

(ii) replacing the wild-type allele of the essential gene with a condition-sensitive homolog thereof which prevents differentiation under the restrictive condition, but not at the permissive condition, to generate a condition-sensitive yeast mutant.

40. The method of claim 39, wherein the condition-sensitive yeast mutant cannot undergo hyphal morphogenesis under the restrictive condition

41. A method for identifying a compound having antifungal activity comprising,

(i) providing a drug screening assay for detecting inhibitors of the function of an essential gene identified by the method according to claim 37 or 39; and

(ii) testing, in the drug screening assay, the ability of a test compound to inhibit the function of the essential gene.

42. A method for treating or preventing an antifungal infection comprising administering to an animal an effective amount of a compound identified according to the method of claim 41 as having antifungal activity.

43. The method of claim 41, comprising the further step of formulating a pharmaceutical preparation including one or more compounds identified as having antifungal activity.

44. A pharmaceutical preparation formulated according to the method of claim 43.

45. A method for identifying a gene or gene product, the expression or stability of which is altered by the function of an essential gene, comprising identifying mRNA transcripts

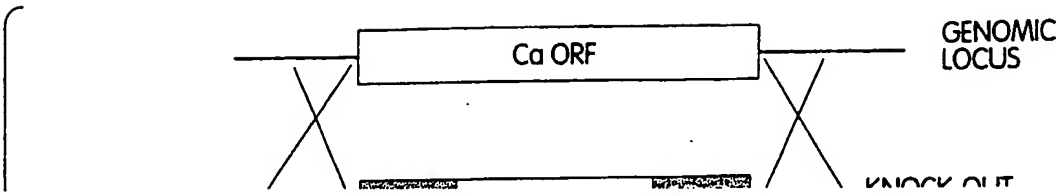
or gene products which have altered levels in a condition-sensitive yeast mutant of claim 19 or 21 under restrictive conditions relative to permissive conditions.

46. The method of claim 45, which method identifies mRNA transcripts which have altered levels under restrictive conditions relative to permissive conditions.

47. A method for identifying a compound having antifungal activity comprising,

(i) providing a drug screening assay for detecting compounds which modulate the function of gene or gene product identified by the method according to claim 45; and

(ii) testing, in the drug screening assay, the ability of a test compound to modulate the function of the gene or gene product.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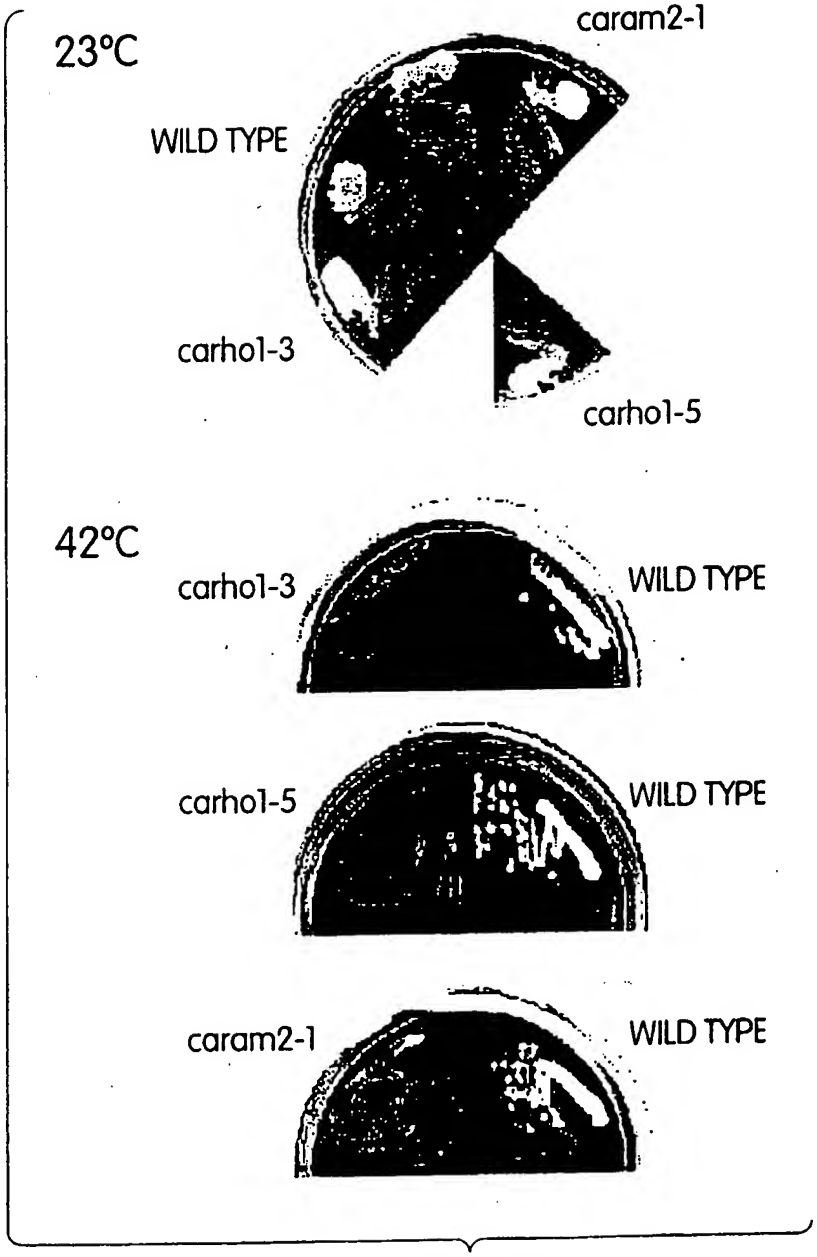
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Fig. 3

# INTERNATIONAL SEARCH REPORT

| | |
|---|---|
| International Application No | PCT/US 00/06514 |

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/81 C12N15/90 C12N15/31 C12N1/19 C12Q1/00
//(C12N1/19,C12R1:725)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, MEDLINE, EPO-Internal, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | OHYA YOSHIKAZU ET AL: "Structure-based systematic isolation of conditional-lethal mutations in the single yeast calmodulin gene." GENETICS, vol. 138, no. 4, 1994, pages 1041-1054, XP000915010 ISSN: 0016-6731 abstract table 2 figure 1 --- -/-- | 34,35 |

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 17 July 2000 | 31/07/2000 |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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| X | SANTOS CRUZ ET AL: "Ribosomal protein P0, contrary to phosphoproteins P1 and P2, is required for ribosome activity and Saccharomyces cerevisiae viability." | 34,35,41 |

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Information on patent family members

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